SUPPLEMENTARY METHODS

Quantitative PCR to test the efficiency of RNAi of PO transcripts/ age-specifc expression of antimicrobial peptides

In our experiments, we closely followed the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines to obtain qPCR data (1). To test the efficiency of RNAi, we first challenged each beetle with peptidoglycan followed after dsRNA injection as described in the main text (see experiment 1). 24 hours later, we harvested 2 µl of hemolymph (collected from a wound between the head and thorax) and abdominal fat body tissue (disseceted as much as possible) from each cold-anesthesized experimental beetle, and suspended immediately in cold Trizol (Sigma). Each RNAi treatment had 8-11 replicates and for each replicate, haemolymph and fat body samples from two individuals were combined and homogenized using a TissueLyser (Qiagen) at 20 Hz for 10 s. We processed the pooled samples (hemolymph and homogenised fat tissues from 2 beetles) immediately to recover RNA using chloroform extraction and isopropanol precipitation according to the manufacturer's instructions. Subsequently, we incubated samples with 2 units of TurboDNase (Ambion) for 30 min at 37°C and isolated RNA using an RNeasy MinElute cleanup kit (Qiagen). We used nanodrop to estimate the quantity and purity of isolated RNA samples. An OD 260/280 ratio between 1.9-2 for all the samples indicated a good quality of isolated RNA. Additionally, we also confirmed the RNA integrity by running it on a 1% standard agarose gel. For all the samples, we ensured that the upper ribosomal band was about twice the intensity of the lower band. We stored the isolated RNA samples at -80°C until further use.

We used 250 ng of pooled total RNA to synthesize cDNA using a cDNA-Synthesis Kit H Plus (Peqlab). Next, we performed qPCR using a peqGOLD Hot Start-Mix kit (Peqlab) with 2.5 ng of cDNA per 15 µl reaction on a StepOne real-time thermocycler (Applied Biosystems) platform according to the manufacturer's instructions. We used Primer3 (2) to design primers for the PO2 gene and the reference gene *rpl27a* that encodes a ribosomal protein (NCBI accession- X99204.1) (Table

S1). We selected rpl27a as a reference gene based on a comprehensive RNAseq dataset that showed its stable expression over 7 days under similar conditions to that of our study (3). Moreover, rpl27a is stable under diverse experimental conditions such as exposure to bacterial infection, sex-difference and aging (Makarova, Davis and Rolff, unpublished data). We used primers described in Dobson et al. (2012) to amplify PO1 transcript (4). We tested each primer for qPCR efficiency, including the reference gene, by regression of Ct values against cDNA concentration. For all the primer pairs, R^2 and amplification efficiency was greater than 0.96 and 90% respectively (4) (see Table S2). We also confirmed that melt curves for all primers had only a single peak. We calculated the relative gene expression as $2^{-\Delta C}{}_{T}$, where ΔC_T is difference in C_T value between gene of interest and ribosomal control gene (5).

We followed similar protocols for RNA isolation from 7-day-old and 42-day-old naïve unhandled beetles and qPCR to estimate age-specific changes in expression of antimicrobial peptides (e.g. *attacin 2* & *tenecin 1*). Each age group had 5-6 replicates and for each replicate, we combined haemolymph and fat body samples from two beetles as outlined above. We used qPCR primers described in Dobson et al. (2012) to amplify *attacin 2* and *tenecin 1* gene (see Table S2 for primer sequences and qPCR efficiency) (4).

A limitation of our qPCR analysis is that we were unable to quantify the relative percentage of fat body in the pooled samples. However, it is unlikely that this would majorly confound our results because of the following reasons- (a) All the beetles were handled similarly to extract haemolymph and fat body samples (b) In experiment 1, it is unclear whether injection of dsRNA of PO alone influences the fat body content differently from mock RNAi treatment with lysozyme from *Galleria mellonella* (c) Finally, in experiment 2, although age may influence fat body content (note that experimental beetles were naïve individuals), but this can also be an integral feature of physiological ageing that affects immunity and hence, lies within the scope of our experimental goals.

SUPPLEMENTARY TABLES

Table S1. T7-tailed primers used for amplification of internal region of cDNA of candidate genes for RNAi.

Gene	**Forward primer** 5'-3'	**Reverse primer** 5'-3'
ProPO1	taatacgactcactatagggagaagaggcgtatttccccaag	taatacgactcactatagggagagattccttcgttctcggtc
ProPO2	taatacgactcactatagggagaaattcttgattctgtagat	taatacgactcactatagggagagagagatcctgtgttctt
Lys	taatacgactcactatagggagagcaagccgaataaaaatgga	taatacgactcactatagggagatatctggcagcggcttattt

Table S2. Primers used for qPCR.

Gene	**Forward primer 5'-3'**	**Reverse primer 5'-3'**	**Efficiency**
*ProPO1**	gcacgagctggaattgtgt	ggtcgaacaacaggaggatg	R^2= 0.96, Amplification efficiency = 90.2%
*ProPO2**	aaaatgcgtagtagaaga	tttatagaagcgaaaaac	R^2= 0.98, Amplification efficiency = 94.2%
rpl27a	tcggaaagttgggaatgagg	tttgaccttgtctgctcact	R^2= 0.98, Amplification efficiency = 98.4%
Attacin 2	tccaccttccatttcgtttc	attcacctctttggcgtttg	R^2=0.97, Amplification efficiency = 93.8.2%
Tenecin 1	ggaagcggcaacagctgaagaaat	aacgcagaccctctttccgttaca	R^2= 0.96, Amplification efficiency = 92.7

Table S3. Summary of Wilcoxon Rank Sum test for relative gene expression in experimental groups- A. RNAi control vs. knockout beetles B. Old vs. young beetles.

	Effect	**Gene**	**χ2**	**DF**	**P**
A.	RNAi	*ProPO1*	11.4714	1	0.001
	n=8-9/treatment/gene	*ProPO2*	12.0079	1	0.001
B.	Age	*Attacin 2*	4.8000	1	0.029
	n=5-6/treatment/gene	*Tenecin 1*	5.6333	1	0.018

Table S4. Summary of (A) ANOVA for phenoloxidase (PO) response with RNAi treatment as a fixed factor and (B) nonparametric Wilcoxon Rank Sum test for Malpighian tubule (MT) activity as a function of RNAi treatments.

A. Trait	**Effect**	**df**	**SS**	**F-ratio**	**P**
PO activity	RNAi	3	0.003	22.552	<0.001
n=16/treatment	Error	60	0.003		
B. Trait	**Effect**	**df**	**χ^2**	**P**	
MT activity	RNAi	3	33.368	<0.001	
n=20-28/treatment					

Table S5. (A) Summary of AFT model, c-parameter estimates and 95% confidence interval for the c parameter (B) Maximum lifespan analysis using exact unconditional z-pooled tests. 'Treatment effect' represents the change in maximum lifespan caused by the treatments (the percentage of survivors to the 90th percentile in the control group/the percentage of survivors to the 90th percentile in the treatment group). C = Unhandled full control beetles, SI = Procedural control for early inflammation (or Sham inflammation), EI = Early inflammation, PO1 = RNAi of PO1 transcript followed by an early-inflammation, PO2 = RNAi of PO2 transcript followed by an early inflammation. Statistically significant comparisons are highlighted in bold.

Assay	Effect	Comparison	Z	P	c-parameter	L-CI	U-CI
A. Median lifespan *n=30/treatment*	Early inflammation	**C vs. EI**	-5.73	**<0.001**	0.655	0.568	0.756
		C vs. PO1	-2.05	**0.04**	0.861	0.746	0.99
		C vs. PO2	-2.02	**0.043**	0.864	0.75	0.99
		C vs. SI	0.06	0.952	1.004	0.869	1.157
		SI vs. EI	-5.79	**<0.001**	0.653	0.565	0.752
		SI vs. PO1	-2.11	**0.034**	0.86	0.744	0.98
		SI vs. PO2	-2.05	**0.038**	0.86	0.746	0.99
	RNAi	**EI vs. PO1**	3.71	**<0.001**	1.31	1.137	1.51
		EI vs. PO2	3.76	**<0.001**	1.32	1.143	1.52

Assay	Effect	Comparison	Treatment effect	Test statistics	P
B. Maximum lifespan *n=30/treatment*	Early inflammation	**C vs. EI**	8.001	-4.75	**0.001**
		C vs. PO1	2.667	-3.09	**0.002**
		C vs. PO2	2.667	-3.09	**0.002**
		C vs. SI	1.143	-0.653	0.538
		SI vs. EI	6.99	-4.21	**0.001**
		SI vs. PO1	2.33	-2.15	**0.032**
		SI vs. PO2	2.33	-2.15	**0.032**
	RNAi	**EI vs. PO1**	0.33	2.01	**0.047**
		EI vs. PO2	0.33	2.01	**0.047**

Table S6. (A) Summary of two-way ANOVA for bacterial clearance with age and time as fixed factors. **(B-C)** Wilcoxon Rank Sum test for the impact of age on (B) antibacterial (AB) activity 1 day (or 7 days) after infection in sham-infected (SI) and infected (I) beetles (C) phenoloxidase (PO) response of naïve beetles. **(D)** Proportional hazard analysis of survival after infection as a function of age and infection status. Statistically significant P values are highlighted in bold.

Trait	Effect	df	SS	F-ratio	P
A. Clearance	Age	1	17.008	13.447	**0.001**
n=9-11/age group/treatment	Time	3	531.946	140.189	**<0.001**
	Age × Time	3	2.892	0.762	0.519
	Error	72	17.171		

Trait	Treatment	Effect	df	$\chi 2$	P
B. AB activity	SI	Age (Day 1)	1	0.404	0.525
n=9-13/age group/treatment		Age (Day 7)	1	3.897	**0.051**
	I	Age (Day 1)	1	5.607	**0.018**
		Age (Day 7)	1	6.29	**0.012**

Trait	Effect	df	$\chi 2$	P
C. PO response	PO activity	1	4.572	**0.032**
n=30/age group				
D. Survival	Infection	1	35.985	**<0.001**
n=14/age group/treatment	Age	1	7.173	**0.007**
	Infection × Age	1	3.01	0.082

Table S7. Summary of (A) Wilcoxon Rank Sum test for Malpighian tubule (MT) activity as a function of age and infection status (B) Wilcoxon Rank Sum test for MT activity of old beetles as a function of RNAi treatments; (C) Proportional hazard analysis of post-infection survival of old beetles as a function of RNAi treatments.

Trait	**Effect**	**df**	**$\chi 2$**	**P**
A. MT activity *n=11-14/age group/infection status*	Ageing and infection	3	28.94	<0.001
B. MT activity *n=12-16/treatment*	RNAi	2	18.679	<0.001
Trait	**Effect**	**df**	**$\chi 2$**	**P**
C. Post-infection survival *n=15-18/treatment*	RNAi	2	18.776	<0.001

SUPPLEMENTARY FIGURES

Figure S1. Reproductive aging of female beetles measured as the mean total number of offspring (±se) produced by each female (24h of mating followed by 72h oviposition period) within 42-days post eclosion. Significantly different groups are indicated by distinct alphabets (ANOVA: DF = 2, SS = 7812.06, F = 21.6343, P <0.001; n = 15-20 beetle/ age-group).

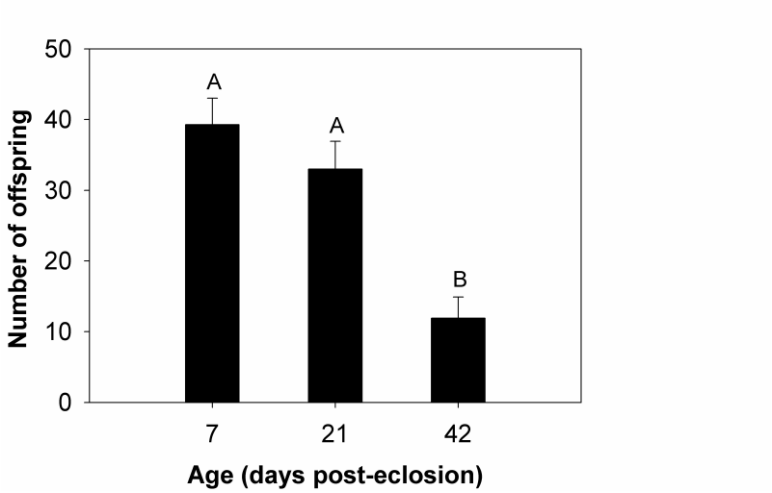


Figure S2. RNAi efficacy of pro-phenoloxidsae transcripts (PO1 and PO2). Significantly different groups are indicated by distinct alphabets. Alphabet assignments are meaningful only within each gene (partitioned by dashed vertical lines), and are not comparable across genes.

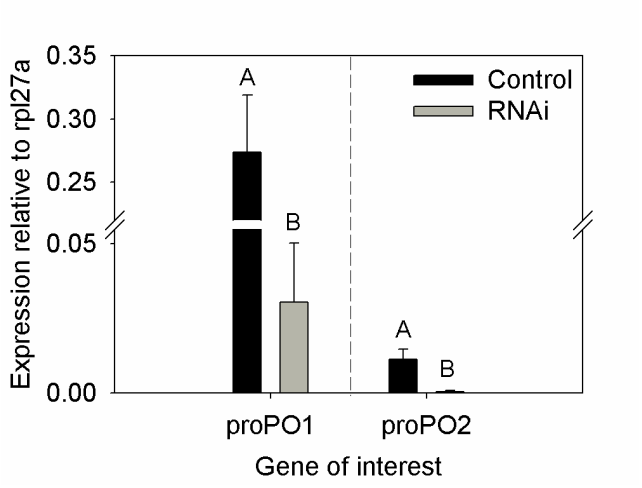
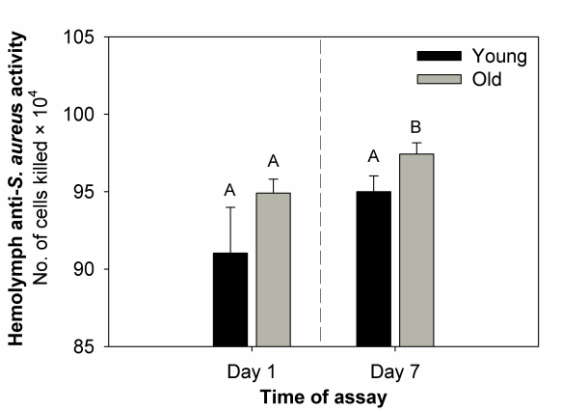


Figure S3. Hemolymph antibacterial activity of sham-infected beetles. Hemolymph antibacterial activity was measured as described in Figure 3 (also see the text). Significantly different groups are indicated by distinct alphabets (based on Steel-Dwass test). Alphabet assignments are meaningful only within each time point (partitioned by dashed vertical lines), and are not comparable across time points.



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